PHYSIOLOGY

Digoxin Facilitates Neuromuscular Transmission in Mouse Diaphragm

O. P. Balezina and V. I. Lapteva

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 144, No. 10, pp. 364-368, October, 2007
Original article submitted January 29, 2007

Low concentration of digoxin (3 nM) facilitated spontaneous and evoked release of neurotransmitter acetylcholine thereby increasing the frequency of miniature end-plate potentials, amplitude of single end-plate potentials, their quantum content and the plateau level in the bursts during stimulation of the phrenic nerve at rates of 4, 7, and 50 Hz. These effects were prevented by blockade of ryanodine receptors with ryanodine (10-20 μM).

Key Words: *motor synapse; ryanodine receptors; transmitter secretion; digoxin; rhythmic activity*

Digoxin, a cardiac glycoside isolated from Digitalis lanata, potentiates the strength of cardiac contractions. The positive cardiotropic effect of digoxin is explained by blockade of the Na^+/K^+-pump in cells [2]. However, other physiological effects of cardiac glycosides and their targets were revealed. Digoxin can enter the cytosol and bind with high affinity to ryanodine receptor (RyR2), which triggers Ca^{2+} release from intracellular stores and elevates cytoplasmic Ca^{2+} concentration. In contrast to direct action of the drug on Na^+/K^+-pump, the intracellular action of digoxin on RyR is realized at very low (nanomolar) concentrations [7,9]. Motor neural terminals contain ryanodine-sensitive Ca^{2+}-depot; Ca^{2+} release from these depots modulates secretion of acetylcholine [6]. However, it remains unknown which type of RyRs (1, 2, or 3) is present in neural motor terminals. In frogs, Ca^{2+}-depot in these terminals contains RyR3 [6]. It is unknown whether RyR2 detected in Ca^{2+}-depot of cardiomyocytes are also present in motor terminals.

Department of Human and Animal Physiology, Biological Faculty, M. V. Lomonosov Moscow State University. ***Address for correspondence:*** balezina@mail.ru. O. P. Balezina

Our aim was to examine the effect of digoxin at low (nanomolar) concentrations incapable to block Na^+/K^+-ATPase (3 nM) on spontaneous and evoked activity of motor synapses in mouse diaphragm.

MATERIALS AND METHODS

The study of spontaneous and evoked synaptic activity in mammalian motor synapses was carried out on isolated mouse neuromuscular preparation *m. diaphragma — n. phrenicus*. The animals were narcotized with ether and decapitated. The left half of the diaphragmatic muscle was isolated with a partion of the phrenic nerve. The isolated preparation was placed into a chamber (3 ml) perfused with oxygenated (95% O_2, 5% CO_2) Laily solution containing (in mM): 135 NaCl, 4.0 KCl, 0.9 NaH_2PO_4, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 16.3 $NaHCO_3$, 11.0 glucose (pH 7.2-7.4). During the experiment, Laily solution was replaced with the test solution, which then persistently perfused the preparation. The experiments were performed at 20°C. Synaptic potentials were recorded intracellularly with glass microelectrodes (resistance 10-15 MΩ, tip diameter ~0.1 μ).

In the control, we recorded membrane potential (MP) of muscle fibers, spontaneous activity of the

0007-4888/07/1444-0487

synapse in the form of miniature end-plate potentials (MEPP), and evoked end-plate potentials (EPP). To record EPP, the split neuromuscular preparation *m. diaphragma — n. phrenicus* was stimulated with suprathreshold pulses (0.3 Hz repetition rate, 0.1 msec pulse duration). Rhythmic activity of the synapse was examined at the frequencies of 4, 7, and 50 Hz. The data were fed to PC via a Digi-Line digitizer equipped with a Digiscope interface and processed with MiniAnalysis software (Synaptosoft). The following parameters were assessed: MP (mV), MEPP discharge rate (Hz), and EPP amplitude (mV).

The data were processed statistically using Student's *t* test in case of normal distribution and Mann—Whitney non-parametric *U* test for independent samples at $p<0.05$.

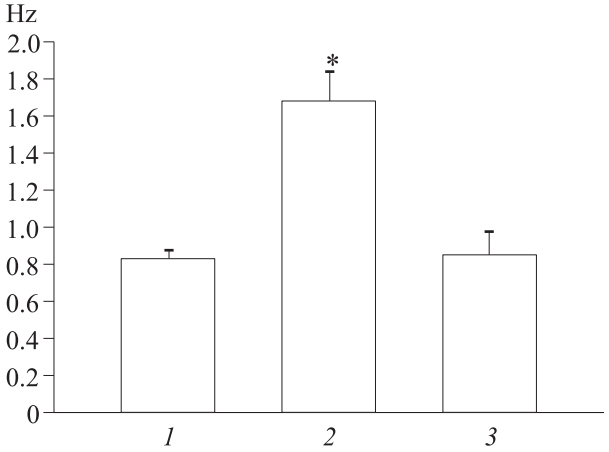

Fig. 1. Effect of digoxin and ryanodine on MEPP frequency in mouse phrenic nerve-muscle preparation. *1*) control; *2*) 3 nM digoxin; *3*) 3 nM digoxin+20 μM ryanodine. Ordinate: MEEP discharge rate. *$p<0.01$ compared to the control value.

RESULTS

In preliminary experiments we found that low concentrations of digoxin (1-10 nM) produced no effect on MP in phrenic muscle fibers (experimental value -71.9±2.7 mV, $n=120$, control value -72.1±2.0 mV, $n=120$). Further increase in digoxin concentrations from 0.1 to 10 μM was associated with a significant decrease in the mean MP from -65.0±1.9 mV to -44.0±1.5 mV ($n=120$; $p<0.001$).

In further experiments we used digoxin in a concentration of 3 nM, which produced no significant effect on MP in phrenic muscle fibers: it remained -71.8±1.2 mV over 30-120-min application. At the same time, digoxin produced a rapid and significant growth of mean discharge rate of MEPP by 2 times as early as 10-15 min after its application to the muscle, which increased from the control level of 0.83±0.05 Hz ($n=86$) to 1.68±0.16 Hz ($n=59$; $p<0.001$). These data suggest that the 2-fold increase in MEPP discharge rate could be caused by the effect of digoxin on the intraterminal RyRs, whose activation results in release of stored Ca^{2+} followed by up-regulation of spontaneous exocytosis of the synaptic vesicles.

To test this hypothesis, the effects of digoxin were studied under conditions of blockade of presynaptic RyRs with the high doses of ryanodine. We found that ryanodine in a concentration of 20 μM did not change MEPP discharge rate over 20-30-min application. Digoxin applied against the background of ryanodine blockade produced no effect on MEPP discharge rate, which was maintained at the control level of 0.85±0.14 Hz ($n=42$, Fig. 1).

The amplitude (A) of individual EPP recorded in some synapses of the dissected phrenic nerve-muscle preparation was A_{EPP}=16.30±0.71 mV ($n=25$), while in the same synapses A_{MEPP} was 0.74±0.04 mV ($n=25$). The quantum content of EPP in the motor synapses of the dissected phrenic nerve-muscle preparation calculated as A_{EPP}/A_{MEPP} ratio was 22±1 (here we used the mean amplitude values).

After 10-15-min application of digoxin to the phrenic nerve-muscle preparation, A_{MEPP} increased by 16.2% to 0.86±0.07 mV ($n=36$, $p<0.05$), while A_{EPP} increased by 25.6% (from the control value of 16.30±0.71 mV to 20.48±1.50 mV). Digoxin produced a small but significant increase of the quantum content of individual EPP by 9% from the control value of 22±1 to 24.00±0.22 ($p<0.05$).

Analysis of short EPP bursts consisting of 50 spikes evoked at a rate of 4, 7, or 50 Hz ($n=69$) revealed typical rate-dependent changes in EPP amplitude during the burst: a drop in EPP amplitude and initial depression of the transmission in the burst at 4 Hz, as well as initial facilitation of the transmission (short-term increment of A_{EPP} at the beginning of the burst, which was small at 7 Hz but pronounced at 50 Hz). A_{EPP} was stabilized to the end of each burst (so called plateau phase of EPP burst). Due to the short-term initial depression of transmission (most expressed at 4 and 7 Hz), EPP amplitude decreased to the end of the burst during the plateau phase in comparison with the amplitude of the first EPP (EPP_1).

Analysis of the effects of digoxin showed that the plateau phase at the end of the burst was most sensitive to digoxin. At stimulation rate of 4 Hz, digoxin significantly increased EPP amplitude during the plateau phase and elevated the $EPP_{plateau}/EPP_1$ ratio to 0.92, which surpassed the control value of 0.87 by 5% ($n=45$, $p<0.05$, Fig. 2, *a*). At stimulation rate of 7 Hz, digoxin increased $EPP_{plateau}/$

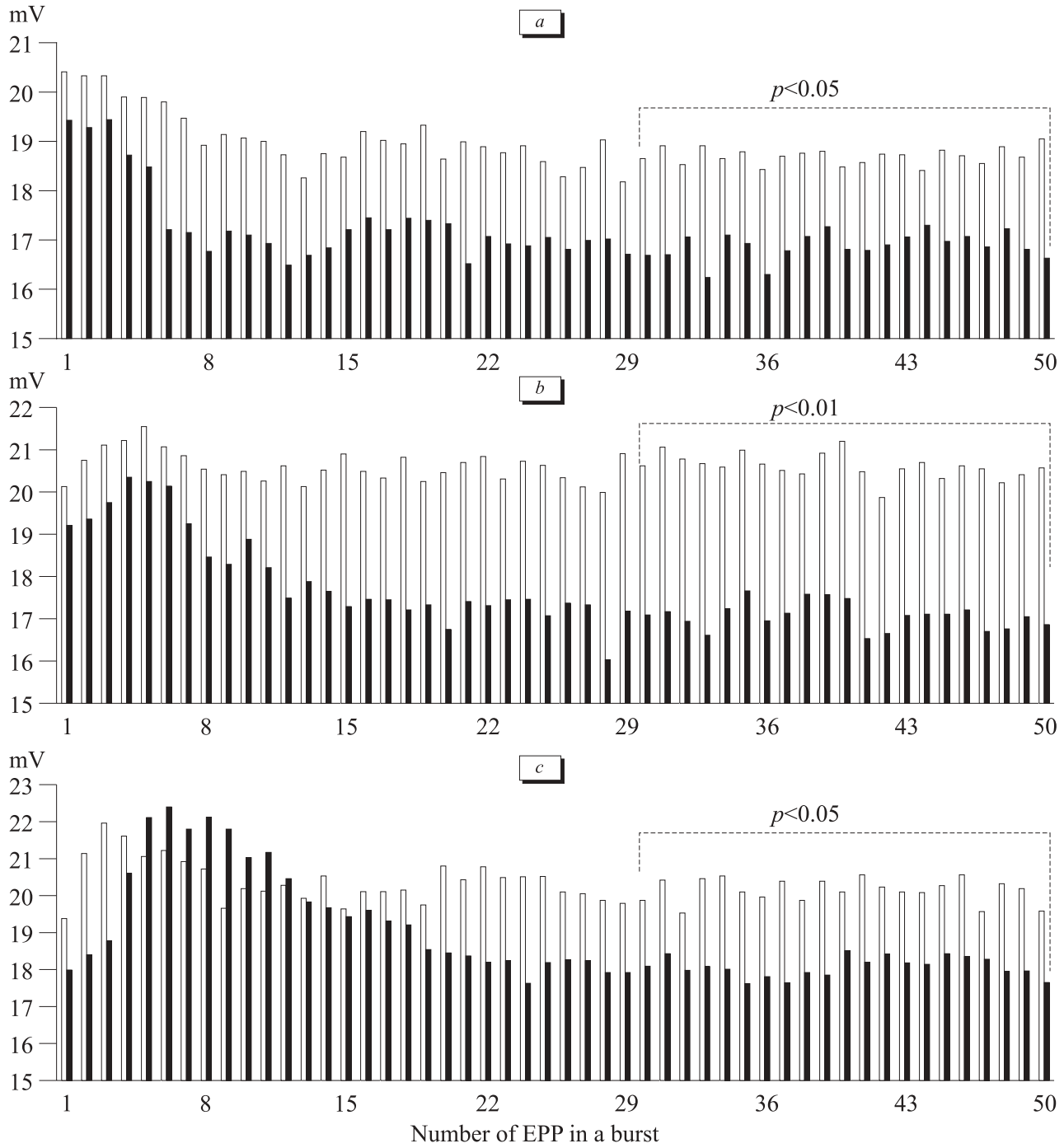

Fig. 2. Effect of digoxin on the amplitude of EPP during rhythmic stimulation of the phrenic nerve at rates of 4 (*a*), 7 (*b*), and 50 Hz (*c*). Dark bars: the control. Open bars: 3 nM digoxin. Average values of EPP in the bursts are shown. $p<0.05$, $p<0.01$ for the last 20 EPP in the burst (the plateau phase).

EPP_1 ratio by 16% to 1.02 from the control value of 0.86 (n=54, $p<0.01$, Fig. 2, *b*). At stimulation rate of 50 Hz, digoxin (n=47) also increased the plateau level in EPP burst. In this case, $EPP_{plateau}/EPP_1$ ratio significantly increased by 4% from 1.00 in the control to 1.04 in the test (Fig. 2, *c*).

For understanding of the nature of digoxin effect on plateau phase amplitude in EPP burst and to assess the ability of digoxin to facilitate evoked activity due to up-regulation of Ca^{2+} release from RyR2-sensitive depot, the digoxin-induced effects were examined in the experiments with preliminary blockade of presynaptic RyRs with ryanodine in high doses.

The facilitating effect of digoxin was most pronounced at a stimulation rate of 7 Hz. Under these

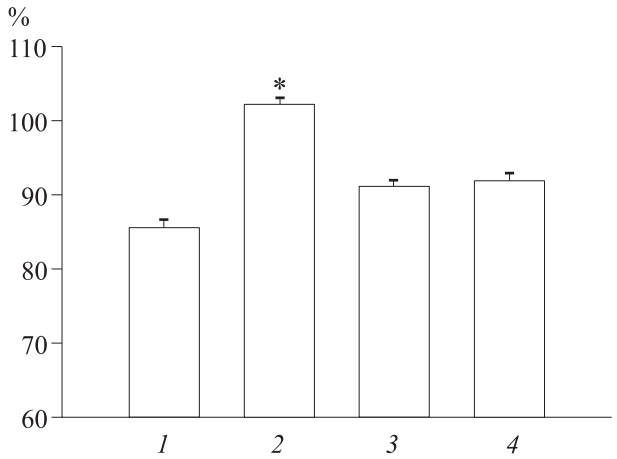

Fig. 3. Effect of digoxin and ryanodine on plateau level in EPP burst at a stimulation rate of 7 Hz. *1*) control; *2*) 3 nM digoxin; *3*) 10 μM ryanodine; 4) 3 nM digoxin+10 μM ryanodine. *$p<0.01$ compared to the control value (*1*).

conditions, application of ryanodine in the blocking concentration of 10 μM significantly increased $EPP_{plateau}/EPP_1$ ratio from the control value of 0.86 to 0.91 (n=46). Addition of digoxin (3 nM) to ryanodine-containing perfusate did not affect EPP amplitude during the plateau phase, which remained at the level observed under individual action of 10 μM ryanodine (Fig. 3).

Thus, our study showed that nanomolar concentrations of digoxin facilitated spontaneous and evoked secretion of acetylcholine quanta in motor synapses. There are published data on the ability of ouabain, another cardiac glycoside that affects synaptic terminals, to facilitate the release of acetylcholine [4,5,10]. Conventionally, the facilitating effects of ouabain and other cardiac glycosides on synaptic transmission and contractile force of cardiomyocytes are explained by their potency to inhibit Na^+/K^+-pump in terminals and to reverse the work of Na^+/Ca^{2+}-tramsporter, thereby enhancing Ca^{2+} level in terminals and up-regulating Ca^{2+}-dependent spontaneous and evoked secretion [4]. However, some effects of cardiac glycosides observed both *in vivo* and *in vitro* cannot be explained entirely by inhibition of Na^+/K^+-ATPase. Therefore, one should consider the possibility of combined action of endogenous and exogenous glycosides on the myocardium and other cells, which includes not only blockade of Na^+/K^+-ATPase, but also intracellular stimulation of RyR2 leading to extra release of stored Ca^{2+} [7,8,12].

Our study showed that digoxin in low (nanomolar) concentrations facilitates the release of neurotransmitter via activation of RyR2 and Ca^{2+} release. Digoxin in low doses produces no effect of Na^+/K^+-pump, which is indirectly corroborated by the fact that low concentrations of digoxin did not decrease MP in muscle fibers within the synaptic region. By contrast, higher concentrations of glycosides (1 μM and higher) inhibit Na^+/K^+-pump and decrease MP of phrenic muscle fibers [1]. Digoxin-induced activation of RyR2 and Ca^{2+} release in the nerve terminals are also attested by the fact that the facilitating effects of the drug were prevented by blockade of RyRs with high concentrations of ryanodine (10-20 μM).

Motor nerve terminals contain a pool of RyRs, whose activation with agonistic caffeine or ryanodine triggers Ca^{2+} release and up-regulation of Ca^{2+}-dependent secretion of the neurotransmitter [11]. This study showed that digoxin, a selective activator of RyR2, could significantly up-regulate spontaneous and evoked secretion of acetylcholine. This phenomenon suggests that not only myocardial, but also the motor terminals possess a functionally significant pool of RyRs controling the release of stored Ca^{2+} and affects neurotransmitter secretion.

REFERENCES

1. T. M. Drabkina and I. I. Krivoi, *Tsitologiya*, **46**, No. 2, 89-104 (2004).
2. *Basic and Clinical Pharmacology* [Russian translation], Ed. B. G. Katzung, Vol. 1, Ìoscow (1998).
3. M. I. Kuzin and M. B. Gekht, *Myasthenia* [in Russian], Moscow (1996).
4. A. S. Pivovarov and D. V. Boguslavskii, *Zh. Vyssh. Nervn. Deyat.,* **50**, No. 5, 855-866 (2000).
5. C. Haimann, F. Torri-Tarelli, R. Fesce, and B. Ceccarelli, *J. Cell Biol.,* **101**, No. 5, Pt. 1, 1953-1965 (1987).
6. M. Kubota, K. Narita, T. Murayama, *et al., Cell Calcium*, **38**, No. 6, 557-567 (2005).
7. S. J. McGarry and A. Williams, *Br. J. Pharmacol*., **108**, No. 4, 1043-1050 (1993).
8. T. Sagawa, K. Sagawa, J. Kelly, *et al., Am. J. Physiol. Heart Circ. Physiol.*, **282**, No. 3, 1118-1126 (2002).
9. R. Sitsapesan, S. J. McGarry, and A. J. Williams, *Trends Pharmacol. Sci*., **16**, No. 11, 386-391 (1995).
10. E. S. Vizi, *Neuroscience*, **3**, Nos. 4-5, 367-384 (1978).
11. D. F. Wilson, *Am. J. Physiol.*, **225**, No. 4, 862-865 (1973).
12. R. Zucchi and S. Ronca-Testoni, *Pharmacol. Rev.*, **49**, No. 4, 1-51 (1997).